Microsecond Luminescence Emission from Copper Cytochrome c[†]

Peter Glatz,* Britton Chance, and Jane M. Vanderkooi

ABSTRACT: The luminescence of Cu-substituted cytochrome c exhibits temperature-dependent decay modes and spectral shifts at temperatures below 80 K. By comparison with the data from metal-substituted porphyrins, we determined intramolecular rate parameters. The observed tripdoublet decay time is 13 ± 1 μs at 77 K and the quartet decay time is 12 ± 5 μs below 30 K. We also investigated the luminescence emission from Cu cytochrome c in the presence of cytochrome c depleted mitochondria and in the presence of soluble cytochrome c oxidase; upon binding, the intensity and decay time of the emission are altered. The results indicate an interaction between cytochrome c and its mitochondrial binding site which depends on the electronic state of cytochrome c.

The luminescence properties of several metal-free or metal-substituted cytochromes c have been characterized in heterogeneous biological systems (Vanderkooi & Erecinska, 1975; Vanderkooi et al., 1973, 1976–1978). The fluorescence qualities have been shown to provide basic information about the interaction of the cytochrome with its mitochondrial binding site, such as the binding (Vanderkooi et al., 1973) and the distance and the orientation of its heme relative to an energy acceptor in cytochrome c oxidase (Vanderkooi et al., 1977). Phosphorescence has also been reported (Vanderkooi et al., 1978a,b) but has not yet been used to characterize the heme and its environment.

The luminescence spectra of copper porphyrins exhibit temperature dependent spectral shifts. These shifts have been interpreted by Gouterman and co-workers to be due to the existence of two excited states, the tripdoublet and the quartet (Ake & Gouterman, 1969; Gouterman, 1973; Gouterman et al., 1970). Since each of these states has its own emission spectrum and lifetime, we expected that the local environment of the porphyrin in cytochrome c could affect each state differentially.

In this paper we report on the steady-state and time-resolved luminescence emission of Cu-substituted cytochrome c. First, we give the intramolecular rate parameters in the temperature region below 80 K, and we relate these parameters to the interaction between the heme and the protein. Second, we compare the data from samples of isolated and of mitochondria-bound copper cytochrome c in freeze-trapped conditions. Since the absorption of the copper in cytochrome oxidase (Chance & Leigh, 1977) overlaps the emission of Cu cytochrome c, energy transfer might occur and could be used to tell distances between the heme of cytochrome c and a copper of cytochrome oxidase.

Experimental Procedure

Steady-State Luminescence Measurement. Luminescence spectra were taken with a spectrofluorometer (Perkin-Elmer MPF-2A) which was modified for the use of a red-sensitive photomultiplier tube (Hamamatsu R636) and interfaced with a desk calculator (Hewlett-Packard 9825A, Cole-Parmer digiphase). The relative spectral sensitivity of the emission monochromator and phototube unit was determined with the fluorescence spectrum of a standard dye (Lippert et al., 1959).

Time-Resolved Luminescence Measurement. A gated flash-lamp (EG & G Fx-124) and a monochromator (Spex minimate) were used for excitation (repetition rate, 0.5 kHz; FW10%M, 1 μs; decay time, 0.6 μs). The phosphorescence light was detected with a red-sensitive phototube (RCA 8852 operated at 2000 V and −70 °C). A cut-off filter (Schott RG630) was set between sample and tube. The lamp gate pulse and the phototube dynode pulse were processed following the single-photon counting technique and time-to-pulse-height conversion (Ortec modules 113, 541, 420A, 436, and TPHC 457). The detection probability of photons was kept below 10%, thus providing undistorted decay curves (Tschanz & Binkert, 1976). The output of the time-to-pulse-height converter was fed into a multichannel analyzer (Lecroy 3001). The measurements were transferred into a desk calculator, plotted, and simulated by using the mathematical model described (see eq 1 and 2).

Simulation of Data. We generated sets of simulated data by using eq 1 and 2 and the experimental lamp flash function (I_a). Let T_i and Q_i be the concentrations of species T and Q at time t_i corresponding to channel i in the multichannel analyzer. Thus

$$\frac{T_{i+1} - T_i}{\Delta t} = I_a\phi_T - k'T_i$$

Letting $\Delta t = 1$ (time increment in 1 channel) then

$$T_{i+1} = I_a\phi_T - k'T_i + T_i \quad (T_0 = 0)$$

Similarly

$$Q_{i+1} = I_a\phi_a + k_{TQ}T_i + k''Q_i + Q_i \quad (Q_0 = 0)$$

The observed luminescence intensity is plotted and compared with the calculated curve:

$$P_i = k_{TS}T_i + k_{QS}Q_i$$

The number of free parameters is reduced because we used the steady-state data of the relative integral intensities of $\sum_{i=1}^{255}T_i$ and $\sum_{i=1}^{255}Q_i$. The free parameters k_{TQ}, $(k_{TS} + k_{TA})$, and $(k_{QS} + k_{QA})$ are found by trial and error optimizing the fit with the least-squares criteria which minimizes

$$\sum_{i=1}^{255}(\text{fit}_i - \text{experiment}_i)^2$$

We simulated the intramolecular relaxation by definite differences ΔT_i, ΔQ_i for all channels i subsequently.

[†] From the Johnson Research Foundation and the Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received February 1, 1979.* This work was supported by U.S. Public Health Service Grant GM 12202. J.M.V. is supported by Career Development Award NIH GM 0053.

0006-2960/79/0418-3466$01.00/0

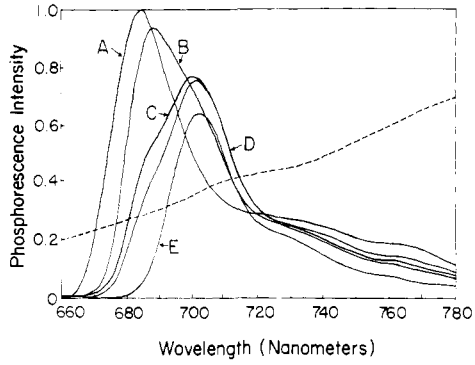

FIGURE 1: Phosphorescence emission of Cu cytochrome *c* (0.1 mM in ethylene glycol–distilled water) at temperatures below 80 K (77 K, curve A; 50 K, B; 33 K, C; 31 K, D; and 12 K, E). The dashed curve is the spectral correction of the detection unit. Excitation wavelength, 400 nm (bandwidth, 20 nm); emission bandwidth, 10 nm. Uncorrected.

Temperature Measurement and Control. The samples were filled into luminescence-free glass tubes (Wilmad Glass NMR tubes). The cooling and temperature control (±2 K) was realized with a helium transfer line and Dewar tube (Air Products and Chemicals) and by measuring the resistance of a charcoal resistor (Scientific Instruments). For the measurements at 77 K, fused quartz round capillary tubes (Bolab Inc.) and a coldfinger liquid nitrogen Dewar were used. The cooling medium for −70 °C was dry ice/ethanol.

Samples. The preparation of iron-free cytochrome *c* from horse heart cytochrome *c* (Sigma Chemical Co., St. Louis, MO) and the binding of metal derivatives of cytochrome *c* to cytochrome oxidase were described by Vanderkooi & Erecinska (1975). The preparation of Cu cytochrome *c* from iron-free cytochrome *c* was established by Vanderkooi et al. (1976). The mitochondria were prepared from rat liver according to the procedure of Chance & Hagihara (1963). The cytochrome *c* depletion of the mitochondria followed the method of Boveris et al. (1972). Cu cytochrome *c* samples were frozen in distilled water/ethylene glycol (1:1 by volume, ethylene glycol from J. T. Baker Chemical Co.); Cu cytochrome *c* bound to rat liver mitochondria was freeze-trapped in a 1:1 mixture of sucrose–phosphate buffer (250 mM sucrose, 10 mM phosphate in distilled water) and distilled water/ethylene glycol. 4,4-Dimethyl-4′-nitrostilbene (Eastman) was used (0.1 mM) in *o*-dichlorobenzene (MCB) for the spectral sensitivity measurement in the range from 560 to 780 nm.

Results

Emission Properties of Isolated Cu Cytochrome c. Luminescence spectra of Cu cytochrome *c* were taken at 200 K and below 80 K. Figure 1 shows the emission of isolated horse heart cytochrome *c* whose iron has been substituted by copper. The emission peak found shifts from 685 to 705 nm as the temperature is lowered from 77 to 12 K and the integrated emission intensity decreases. In the region from 35 to 30 K, we observed the continuous disappearance of the 689-nm peak and the appearance of the 705-nm peak. The excitation band at 400 nm (Soret absorption band) broadens and shifts to 390 nm (Figure 2). The excitation bands above 500 nm do not change in shape or position. We did not detect any free porphyrin fluorescence emission.

The left side of Figure 3 shows the diagram which was established by Gouterman (1973) for metal substituted porphyrins with one unpaired d electron. By comparison with the Cu mesoporphyrin emission data (Gouterman et al., 1970), we assigned the band with the temperature-dependent peak

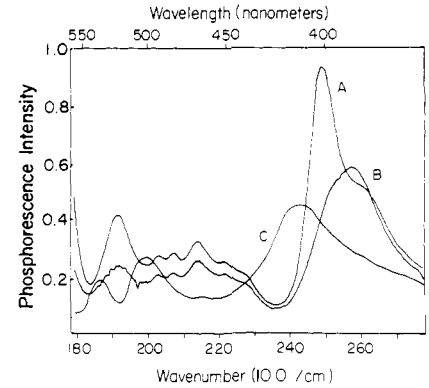

FIGURE 2: Phosphorescence excitation spectra of Cu cytochrome *c* emitting at 685 nm (curve A, temperature 50 K) and at 705 nm (curve B, temperature 11 K). The band widths are 20 nm (emission) and 10 nm (excitation). The excitation spectrum of protoporphyrin (in dimethylformamide) emitting at 700 nm (at 77 K) is shown in curve C. Uncorrected.

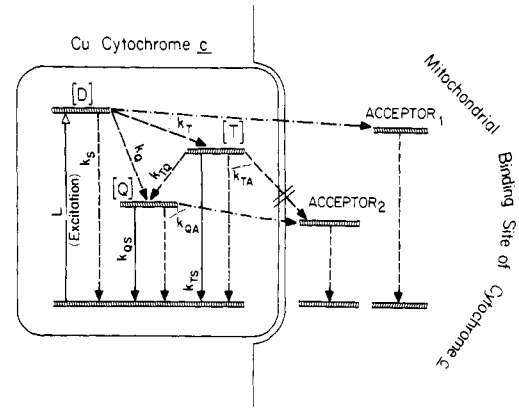

FIGURE 3: The energy diagram (left side) of metal-substituted porphyrins (from Gouterman and co-workers) was applied to explain the phosphorescence emission characteristics of Cu cytochrome *c*. Possible energy-transfer interactions between the enzyme and its mitochondrial binding site are shown with arrows to the right side. Acceptor$_1$ is assumed to be a heme (4) and acceptor$_2$ a copper of cytochrome oxidase (9).

position to the radiative tripdoublet deexcitation and the band which appears below 35 K to the radiative quartet deexcitation. The energy gap between tripdoublet and quartet (calculated from the peak wavelengths) decreases from 0.051 eV at 77 K to 0.038 eV at 25 K.

We describe the population of tripdoublet and quartet levels as follows: ground state [D_0], doublet [D], tripdoublet [T], and quartet [Q]. I_a stands for the absorbed light intensity.

$$[D_0] + h\nu \rightarrow [D] \rightarrow [T] \qquad \text{rate: } I_a\phi_T$$
$$\searrow [Q] \qquad I_a\phi_Q$$
$$[T] \rightarrow [Q] \qquad k_{TQ}[T]$$
$$[T] \rightarrow [D_0] + h\nu' \qquad k_{TS}[T]$$

(radiative, emission 685–689 nm)

$$[T] \rightarrow [D_0] + \text{heat} \qquad k_T[T]$$
$$[Q] \rightarrow [D_0] + h\nu'' \qquad k_{QS}[Q]$$

(radiative, emission 705 nm)

$$[Q] \rightarrow [D_0] + \text{heat} \qquad k_Q[Q]$$

With the following simplifying assumptions, the differential equation system can be given and solved easily.

Assumption 1: The tripdoublet (symbol T) and the quartet (Q) are immediately populated from the doublet (D) with efficiencies T and Q, respectively.

Assumption 2: The back rate quartet → tripdoublet can be neglected and the population of the tripdoublet from the

Table I: Molecular Rate Parameters of Cu Cytochrome *c*

rate parameter (10^4/s)	temp (K)					
	200	77	45	25	10	7
$k_{TS} + k_T$[a]	110 ± 12	7.6 ± 0.5[d] 6.5 ± 0.5[e]	7.6 ± 0.5	7.6 ± 0.5	<0.5	4.8 ± 1
$k_{QS} + k_Q$[b]		9 ± 2	6 ± 2	5 ± 2	7 ± 2	10 ± 2
k_{TQ}[c]		0.3 ± 0.03	1 ± 0.1	122 ± 10	105 ± 10	5.4 ± 1

[a] Tripdoublet decay (tripdoublet to quartet crossing subtracted). [b] Quartet decay. [c] Tripdoublet to quartet crossing. [d] Isolated Cu cytochrome *c*. [e] Cu cytochrome *c* in the presence of mitochondria.

excited doublet, $D_1 \rightarrow T$, is at least 1000 times more efficient than the population of the quartet, $D_1 \rightarrow Q$. If k is the Boltzman constant, T the temperature, and E_{TQ} the energy gap between T and Q, the ratio ϕ_Q/ϕ_T between the population rates of Q and T from D is equal to $\exp(-E_{TQ}/kT)$ which can be neglected compared with unity in the entire temperature range considered here since E_{TQ}/kT is >6 if $T < 100$ K.

Both assumptions refer to the theory for the luminescence states in porphyrin systems (Ake & Gouterman, 1969).

The differential equations describing the tripdoublet and quartet populations are

$$d[T]/dt = I_a\phi_T - k'[T] \quad (1)$$

$$d[Q]/dt = I_a\phi_Q + k_{TQ}[T] - k''[Q] \quad (2)$$

$$(\text{with } k' = k_{TQ} + k_{TS} + k_T,\ k'' = k_{QS} + k_Q)$$

The solutions are

$$[T] = I_a\phi_T \exp(-k't) \quad (3)$$

$$[Q] = I_a\phi_Q \exp(-k''t) + (k_{TQ}I_a\phi_T/(k'' - k'))(\exp(-k't) - \exp(-k''t)) \text{ (if } k' \neq k'') \quad (4a)$$

$$[Q] = (I_a\phi_Q + k_{TQ}I_a\phi_T t)\exp(-k''t) \text{ (if } k'' = k') \quad (4b)$$

For continuous (steady-state) excitation, the ratio between tripdoublet and quartet plus tripdoublet emission is

$$k_{TS}[T]/(k_{QS}[Q] + k_{TS}[T]) = 1/(1 + k_{TQ}/(k_{TS}(1 + k_Q/k_{QS}))) \quad (5)$$

From the spectra and decay properties of emission, the rate parameters k_{TQ}, k', and k'' can be determined.

The intensity ratio between the tripdoublet and the total emission was determined by fitting the spectrum at a given temperature with the emission spectrum of the quartet at 15 K and tripdoublet at 77 K. The intensity ratio is given on Figure 4. The integral intensity of the tripdoublet decreases with decreasing temperature, while the quartet emission intensity first increases and then decreases with the temperature. The quartet intensity is maximal at 33–31 K.

Phosphorescence Decay of Isolated Cu Cytochrome c. From the data shown in Figure 4, we determined the ratio between tripdoublet to quartet intersystem crossing k_{TQ} and apparent radiative tripdoublet deexcitation k_{TS} ($(1 + k_Q)/k_{QS}$) as a function of the reciprocal temperature (Figure 5). A $k_{TQ}/[k_{TS}((1 + k_Q)/k_{QS})]$ value of more than 1 below 30 K reflects the enhanced population of the quartet level from the tripdoublet due to the decreased energy gap E_{TQ}. The difference in activation energy of k_{TQ} and $k_{TS}(1 + k_Q/k_{QS})$ is 0.014 eV per molecule which is 30–40% of the energy gap itself.

Decay measurements provide direct information about the rate for deexcitation from the tripdoublet. We found single-exponential and nonexponential decay modes above and below 30 K, respectively (Figure 6), with decay times of the order of 10 μs. Temperature-dependent rate constants and nonexponential decays are predicted if the quartet is populated from the tripdoublet (eq 4).

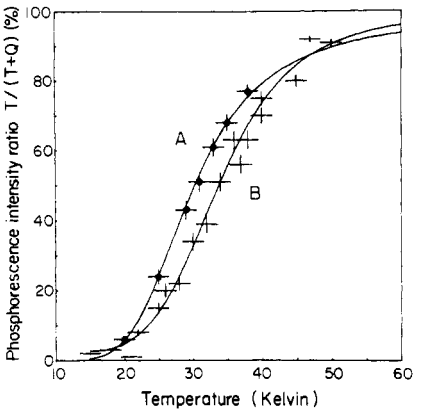

FIGURE 4: The ratio of tripdoublet T to total (tripdoublet plus quartet) phosphorescence intensity T + Q of Cu cytochrome *c* as a function of the temperature. Isolated Cu cytochrome *c*, curve A; rat liver mitochondria bound Cu cytochrome *c*, curve B. The fits are determined with ln (Q/T), least-squares fit as a function of the reciprocal temperature (see Figure 5).

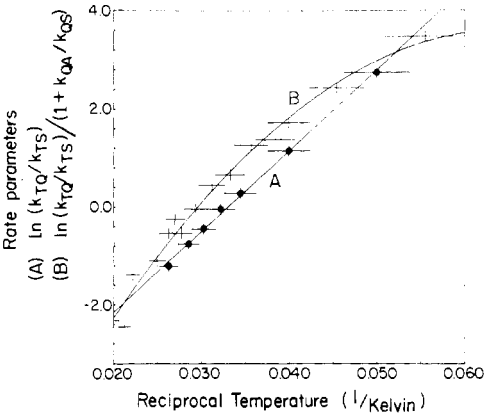

FIGURE 5: Relationship between intramolecular (k_{TQ}, k_{TS}, and k_{QS}) and intermolecular rates (k_{TA}) and the reciprocal temperature. The data points correspond to Figure 4 for isolated (filled circles) and membrane-bound Cu cytochrome *c* (open circles) and have been calculated with eq 5–7. Curve A is the linear least-squares fit of ln $(Q/T) = -5.4 + 0.014$ eV$/kT$ and curve B is ln $(Q/T) = -8.8 + 3.8 \times 10^2/T - 2.9 \times 10^3/T^2$. Q denotes the quartet luminescence intensity.

From the time-resolved measurements we determined the rate parameters as shown in Table I.

Altered Emission Characteristics in the Presence of Mitochondria. The comparison between isolated and mitochondria-bound cytochrome *c* provides a source of information on the interaction between the cytochrome and its binding site. Therefore, it is significant that the spectral properties taken from rat liver mitochondria bound Cu cytochrome *c* are different from the spectra found with the isolated enzyme in two respects. First, the integrated intensity of phosphorescence

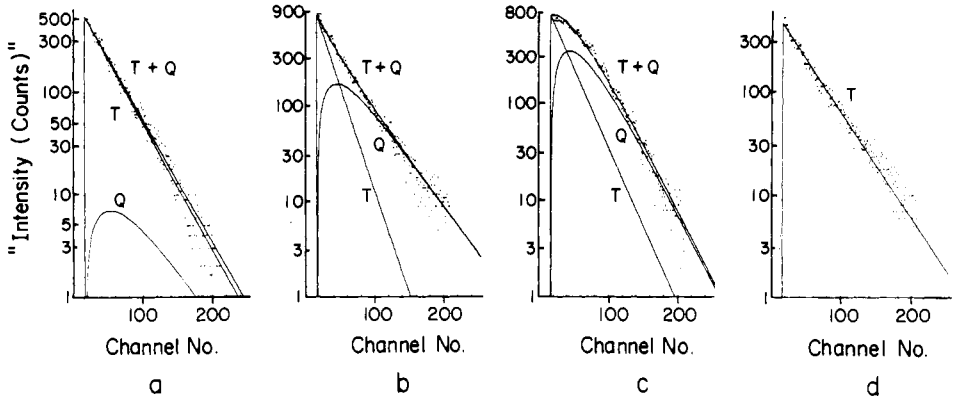

FIGURE 6: Phosphorescence decay of Cu cytochrome *c* (in ethylene glycol–distilled water) at temperatures 77 K (a), 25 K (b), and 7 K (c). Curve d is the decay of Cu cytochrome *c* bound to rat liver mitochondria at 77 K. Excitation wavelength, 400 nm (band width, 20 nm); emission filter, Schott RG630; photomultiplier, RCA 8852 operated at −70 °C and 2000 V. The time base calibration is 0.37 ± 0.01 μs/channel.

from cytochrome *c* is lower and, second, the ratio between tripdoublet to total luminescence is altered (Figure 4). Additionally a longer decay time at 77 K and nonexponential decay was observed (Figure 6d). This was found for Cu cytochrome *c* in the presence of cytochrome *c* depleted mitochondria and in the presence of soluble cytochrome oxidase.

The observation that the ratio $k_{TQ}/[k_{TS}((1 + k_Q)/k_{QS})]$ (all are intramolecular rate parameters) is not exponential with the reciprocal temperature in the presence of mitochondria is not compatible with the simple energy level and rate parameter scheme used for the isolated cytochrome *c*. Therefore, we also consider tripdoublet and/or quartet quenching (e.g., due to energy transfer from cytochrome *c* to an energy acceptor at the binding site; Figure 3, right side).

The steady-state solution of a more general equation system yields for the tripdoublet to total luminescence intensity ratio

$$k_{TS}[T]/(k_{QS}[Q] + k_{TS}[T]) = 1/(1 + fk_{TQ}/k_{TS}) \quad (6)$$

with f, a function of the intramolecular rate parameters and intermolecular energy-transfer probabilities. With the assumptions stated in the first part of the Results

$$f = 1/(1 + (k_Q + k_{QA})/k_{QS}) \quad (7)$$

with k_{QA}, a rate parameter describing the cytochrome *c* to binding site interaction. $(k_Q + k_{QA})$ turns out to be negative (from Figure 5), and the luminescence decay of bound cytochrome *c* is not shortened at 77 K (Figure 6d). Therefore, we must exclude the possibility of a dominant energy transfer from the splitted triplet level (i.e., tripdoublet and quartet) at 77 K. It is significant from the data shown in Figure 5 that the binding of Cu cytochrome *c* to mitochondria affects preferentially the quartet emission. Possible mechanisms are presented in the Discussion.

Discussion

A simple reaction scheme for different electronic states of isolated Cu cytochrome *c* has been used to analyze time-resolved and steady-state luminescence data. We conclude that the theory of metal-substituted porphyrins with an unpaired d electron is applicable. From the comparison between free Cu porphyrin emission data (Ake & Gouterman, 1969; Gouterman, 1973; Gouterman et al., 1970; Gouterman & Khalil, 1974; Smith & Gouterman, 1968) and our results, it is obvious that the observed decay rates are enhanced in cytochrome *c*.

The luminescence decay rates of isolated metal-free and metal-substituted porphyrins are one to two orders of magnitude smaller than of this metal-substituted cytochrome *c*: copper mesoporphyrin tripdoublet luminescence decays within 97 μs at 74 K, 1.3 ms at 11 K, and its quartet luminescence is emitted within 72–145 μs. VO tetraphenylporphyrin is the fastest decaying porphyrin which is reported with a metal having a single unpaired electron with decay times of 16 μs at 81 K and 8 μs at 23 K, but its emission spectrum is not influenced by a temperature change. Therefore, a reason for the enhanced rates of a protein-linked porphyrin such as Cu cytochrome *c* must be found which is not applicable to frozen solutions of free porphyrins.

The metal-substituted porphyrin (originally a heme) is covalently bound to and embedded in a protein. There are several mechanisms with which the polypeptide chain can influence the luminescence characteristics. First, the covalent linkages provide a firm coupling between the porphyrin edge and the protein chain. Therefore, the protein may enhance the vibrational relaxation of the excited porphyrin. We find indeed less structured bands and larger band widths than reported of free porphyrins. Second, the amino acids which form the heme crevice (an opening in the protein core) are positively charged. Therefore, the porphyrin is surrounded by a ring-like positive charge distribution which can influence its electrical charge density in both the tripdoublet and the quartet state. Third, the ligand on the copper atom may affect the decay rate. It has been shown by paramagnetic resonance (Chien et al., 1977) that the copper is liganded to a nitrogen and a sulfur in copper cytochrome *c*.

Mitochondrial bound Cu cytochrome *c* emission shows two differences compared with the isolated enzyme: the parameter ratio $k_{TQ}/[k_{TS}((1 + k_Q)/k_{QS})]$ (Figure 5) is nonexponential as a function of $1/T$ and the overall intensity is diminished. From fluorescence emission data (Vanderkooi et al., 1977, 1978a,b), we conclude that the doublet energy overlaps with the absorption spectrum of cytochrome oxidase in the 600-nm region. Therefore, doublet quenching may occur and it explains the decreased luminescence intensity observed for bound cytochrome *c*.

One of the possibilities of energy transfer between Cu cytochrome *c* and cytochrome oxidase is dipole–dipole interaction. The distance for resonance energy transfer from the quartet of Cu cytochrome *c* to the 700-nm absorbing acceptor (Chance & Leigh, 1977; Van Buuren et al., 1972; Camerino & King, 1966) in cytochrome oxidase (attributed to copper) can be estimated to be 1.5–1.8 nm. This is based upon Förster's (1951) theory of energy transfer; the assumption is made that energy transfer from the split triplet state of porphyrin to a copper of cytochrome *c* oxidase is not spin-forbidden. The distance from the center of the heme of cytochrome *c* to copper is thus estimated to be less than the distance between heme *c* and heme *a* or a_3 (Vanderkooi et al.,

1977). The above distance estimation includes a correction for short distance transfer (see Experimental Procedure and the Appendix) and assumes a random orientation of the heme *c* relative to copper.

Acknowledgments

We thank Dr. A. J. Waring, A. G. Bonner, J. R. Sorge, and G. V. Woodrow for advice and assistance in the mechanical, electronical, and preparative parts of this work.

Appendix

The electrostatic field which is produced by an electric dipole $q_m\mathbf{m}$ and seen by an immobile dipole $q_n\mathbf{n}$ is

$$E(q_m\mathbf{m}, q_n\mathbf{n}, \mathbf{r}) = \frac{q_m q_n}{4\pi\epsilon_0}\left[\frac{((\mathbf{r}-\mathbf{m})/2)\mathbf{n}}{|(\mathbf{r}-\mathbf{m})/2|^3} - \frac{((\mathbf{r}+\mathbf{m})/2)\mathbf{n}}{|(\mathbf{r}+\mathbf{m})/2|^3}\right]$$

where $\mathbf{r}$ is the distance vector from $\mathbf{m}$ to $\mathbf{n}$, q_m and q_n are the charges and ϵ_0 is the dielectric constant. We calculate

$$f = \left[\frac{((\mathbf{r}-\mathbf{m})/2)\mathbf{n}}{|(\mathbf{r}-\mathbf{m})/2|^3} - \frac{(\mathbf{r}+\mathbf{m})/2)\mathbf{n}}{|(\mathbf{r}+\mathbf{m})/2|^3}\right]^2$$

for different relative orientations of $\mathbf{m}$, $\mathbf{n}$, and $\mathbf{r}$. ($\mathbf{m}$ and $\mathbf{n}$ are assumed to be unit vectors, and $r = |\mathbf{r}|$.)

(a) $\mathbf{m}$, $\mathbf{n}$, and $\mathbf{r}$ are parallel ($\mathbf{mr} = r = \mathbf{nr}$ and $\mathbf{mn} = 1$);

(b) $\mathbf{m}$ and $\mathbf{n}$ are parallel, and $\mathbf{r}$ is perpendicular to $\mathbf{m}$ and to $\mathbf{n}$ ($\mathbf{mr} = 0 = \mathbf{nr}$ and $\mathbf{mn} = 1$);

(c) $\mathbf{m}$ and $\mathbf{n}$ are perpendicular, and $\mathbf{r}$ and $\mathbf{n}$ are parallel ($\mathbf{mr} = 0 = \mathbf{mn}$ and $\mathbf{nr} = r$);

(d) $\mathbf{m}$ and $\mathbf{n}$ are perpendicular, and $\mathbf{r}$ and $\mathbf{m}$ are parallel ($\mathbf{nr} = 0 = \mathbf{mn}$ and $\mathbf{mr} = r$);

(e) $\mathbf{m}$ and $\mathbf{n}$ are perpendicular, and $\mathbf{r}$ is perpendicular to $\mathbf{m}$ and to $\mathbf{n}$ ($\mathbf{mn} = \mathbf{mr} = \mathbf{nr} = 0$).

The results are

(a) $f = 1/[(r - 1/2)^2 - 1/(r + 1/2)^2]^2$;

(b) $f = 1/(r^2 + 1/4)^3$;

(c–e) $f = 0$ (no electrostatic energy).

The f function now replaces the ratio between the orientation factor $\mathcal{H}^2 = (\mathbf{mn} - 3(\mathbf{mr}/\mathbf{r})(\mathbf{nr}/\mathbf{r}))^2$ and the sixth power of the distance r in the Förster equation for resonance energy transfer (Förster, 1951).

The five relative orientations a–e are averaged taking into account their individual statistical weights in a random distribution. Situation a has the statistical weight 1/9; the other situations have 2/9. The averaged f function then is

$$f = 1/[(r - 1/2)^2 - 1/(r + 1/2)^2]^2 9 + 2/(r^2 + 1/4)^3 9$$

The averaging procedure corresponds to the substitution of $\mathcal{H}^2$ with 2/3.

References

Ake, R. L., & Gouterman, M. (1969) *Theor. Chim. Acta 15*, 20–42.

Boveris, A., Erecinska, M., & Wagner, M. (1972) *Biochim. Biophys. Acta 256*, 223–242.

Camerino, P. W., & King, T. E. (1966) *J. Biol. Chem. 241*, 970–979.

Chance, B., & Hagihara, B. (1963) *Proc. Int. Congr. Biochem., 5th*, 3–37.

Findlay, M. C., Dickinson, L. C., & Chien, J. C. W. (1977) *J. Am. Chem. Soc. 99*, 5168–5173.

Förster, Th. (1951) in *Fluoreszenz Organischer Verbindungen*, pp 67–86, Vandenhoeck, Göttingen.

Gouterman, M. (1973) in *Excited States of Matter* (Shoppee, Ed.) pp 63–103, Graduate Studies, Texas Tech University.

Gouterman, M., & Khalil, G. E. (1974) *J. Mol. Spectrosc. 53*, 88–100.

Gouterman, M., Mathies, R. A., Smith, B. E., & Caughey, W. S. (1970) *J. Chem. Phys. 52*, 3795–3802.

Lippert, E., Nagele, W., Seibold, I., & Staiger, U. (1959) *Z. Anal. Chem. 170*, 1–17.

Smith, B. E., & Gouterman, M. (1968) *Chem. Phys. Lett. 2*, 517–519.

Tschanz, H. P., & Binkert, Th. (1976) *J. Phys. E 9*, 1131–1136.

Van Buuren, K. J. H., Nicholls, P., & Van der Gelder, B. F. (1972) *Biochim. Biophys. Acta 256*, 258–276.

Vanderkooi, J. M., & Erecinska, M. (1975) *Eur. J. Biochem. 60*, 199–207.

Vanderkooi, J. M., Erecinska, M., & Chance, B. (1973) *Arch. Biochem. Biophys. 154*, 219–229.

Vanderkooi, J. M., Adar, F., & Erecinska, M. (1976) *Eur. J. Biochem. 64*, 381–387.

Vanderkooi, J. M., Landesberg, R., Hayden, G. W., & Owen, C. S. (1977) *Eur. J. Biochem. 81*, 339–347.

Vanderkooi, J. M., Chance, B., & Waring, A. (1978a) *FEBS Lett. 88*, 273–274.

Vanderkooi, J. M., Leigh, J. S., Owen, C. S., Glatz, P., & Blum, H. (1978b) in *Frontiers in Biological Energetics* (Scarpa, Dutton, & Leigh, Eds.) pp 54–62, Academic Press, New York.